

SCHOOL OF APPLIED SCIENCES

ENV10112 Scientific Research Project Honours Thesis

Project Title	The Effect of Pro-Inflammatory IL-1 α on <i>FSCN1</i> Expression in A549 Cells – A Potential Link between Acute Respiratory Distress Syndrome and Non-Small Cell Lung Cancer Metastasis
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1. ABSTRACT

Lung cancer is the most lethal cancer in the UK and worldwide, with most cases being non-small cell lung cancer. Fascin-1 (encoded by *FSCN1*) is an actin-binding protein promoting cell migration and metastasis by stabilising associated protrusive structures in cancer cells. IL-1 β has been shown to increase *FSCN1* expression, but pro-inflammatory IL-1 α is less explored. In acute respiratory distress syndrome, IL-1 α can act as a damage-associated molecular pattern when alveolar epithelial cells necrotise. Here, the aim is to investigate the effects of IL-1 α on A549 cells as a model for non-small cell lung cancer. RNA was extracted from A549 cells and checked for quality before reverse transcribing to obtain cDNA. To check cDNA integrity and normalise *FSCN1* expression, the housekeeper *ACTB* (β -actin) was used. *FSCN1* primers were optimised via an annealing temperature gradient and the end-point PCR products were gel electrophoresed. An unpaired two-tailed t-test was applied to densitometric data of the amplicons to determine significance. RNA quality was deemed good and reverse transcription was successful with clear *ACTB* amplicons on the agarose gel. Furthermore, *FSCN1* amplicons appeared at all temperatures on the gradient. Five total repeat groups were tested comparing untreated and IL-1 α treated A549 cells. A minor but non-significant decrease in *FSCN1* expression in IL-1 α treated A549 cells was observed. Future investigations should therefore look at transient IL-1 α treatment at higher concentrations. Furthermore, other cancer cell lines should also be explored.

2. INTRODUCTION

2.1. The Significance of Lung Cancer

With 13% of new cancer cases between 2016 and 2018, lung cancer is the third most common cancer in the United Kingdom (Cancer Research UK, n.d.). Lung cancer is also the leading cause of death due to cancer in the United Kingdom with 34,771 deaths between 2017 and 2019 (Cancer Research UK, n.d.; Herbst et al., 2018). Only 10% of lung cancer patients in England between 2013 and 2017 survive more than 10 years; globally, 1.6 million deaths due to lung cancer are recorded yearly (Cancer Research UK, n.d.; Torre et al., 2015). Furthermore, the economic

burden of lung cancer treatment in the United Kingdom is heavy at £2.4 billion annually (The National Cancer Research Institute, 2012). While the cost for treatment per patient for prostate cancer is £1,584 per year, the cost for lung cancer is much higher at £9,071 per year (The National Cancer Research Institute, 2012). As such, it is warranted that more research is conducted on lung cancer, its causes and appropriate treatment options.

Lung cancer can be split into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) - NSCLC makes up 85% of cases (Herbst et al., 2018). NSCLC can be further subdivided, with the lung adenocarcinoma and squamous cell carcinoma subtypes being the most common (Herbst et al., 2018). Lung adenocarcinoma cases make up 40% of all NSCLC cases (Travis et al., 2015). As such, it is unfortunate that specifically NSCLC survival rates remain low, especially when metastasis occurs (Herbst et al., 2018). Both stage I and II are usually treated surgically; however, there is an increased risk of developing secondary lung cancers (Duma et al., 2019).

Besides smoking, there is also a consensus that chronic inflammation plays a role in lung cancer (O'Callaghan et al., 2010). This includes various causes of chronic inflammatory diseases like chronic obstructive pulmonary disease (COPD), air pollutants, and pulmonary infections like tuberculosis (O'Callaghan et al., 2010). Acute inflammation, as is the case in acute respiratory distress syndrome (ARDS), and its effects on cancer seems to have been less extensively explored to date.

2.2. The Pathophysiology of Acute Respiratory Distress Syndrome (ARDS)

Acute respiratory distress syndrome (ARDS) is an acute and diffuse inflammatory condition of the lungs leading to respiratory failure (Fan et al., 2018). ARDS is often the result of respiratory infections, of which the recent COVID-19 pandemic is a prominent example (Attaway et al., 2021). Indeed, approximately 15-30% of patients hospitalised with COVID-19 developed ARDS (Attaway et al., 2021). Such lung injury will lead to inflammatory damage to the alveolar-capillary barrier and increased vascular permeability (Batah & Fabro, 2021). Ultimately, this will compromise gas exchange and will lead to hypoxemia and, as such, ARDS (Batah & Fabro, 2021). More specifically, the Berlin definition of ARDS outlines that three criteria must be

met for ARDS (Fanelli et al., 2013). These are acute respiratory symptoms, bilateral infiltration in the chest on X-ray images, and hypoxemia (Fanelli et al., 2013). Beyond endothelial cell damage, epithelial cell damage also occurs as a precursor to ARDS (Batah & Fabro, 2021; Matthay et al., 2019). As mentioned, this damage can be direct by viral or bacterial infections, or also indirect by inflammatory cells and their products (Matthay et al., 2019). Such damage has also been observed to be exacerbated by ventilators through ventilator-associated lung injury (VALI) (Matthay et al., 2019). The alveolar epithelium is composed of flat alveolar type I (ATI) and cuboidal alveolar type II (ATII) cells – these form barriers, but allow for oxygen and carbon dioxide diffusion (Matthay et al., 2019). Both necrotic and apoptotic cell death of ATI/II are key characteristics in ARDS – furthermore, necrosis releases damage-associated molecular patterns (DAMPs) like IL-1 α into the alveolar space (Dinarello, 2018; Matthay et al., 2019).

2.3. The Role of Fascin-1 in Cancer

Fascin-1 is an actin-binding globular 493 amino acid long protein of approximately 55 kDa (Hashimoto et al., 2011; Liu et al., 2021). The 5-exon gene expressing fascin-1 – *FSCN1* – is located on chromosome 7p22.1 in humans (Liu et al., 2021). *FSCN1* is highly homologous and conserved across multiple species, and as such, this suggests a critical biological function (Liu et al., 2021). Fascin-1 contains four tandem domains (β 1-4) with a β -trefoil folding motif, where each fascin-1 molecule will bind two actin molecules (Hashimoto et al., 2011). Fascin-1 supports cellular structures such as filopodia, microspikes, and other protrusions – all of which are needed for cell migration (Darnel et al., 2009; Hashimoto et al., 2005). This support of cellular structures by fascin-1 plays an important role in embryonic development (Liu et al., 2021). Because of this, most normal adult cells will not express fascin-1 except for fascin-1 being involved in tissue repair (Hashimoto et al., 2011; Qualtrough et al., 2011). However, fascin-1 levels are altered in many tumours, and as such, fascin-1 has become a carcinoma marker in many tissue types (Hashimoto et al., 2011). Indeed, fascin-1 has been found to promote cell migration and metastasis (Darnel et al., 2009). Furthermore, the expression of fascin-1 seems to correlate with a decreased overall survival rate (Hashimoto et al., 2011). Silencing of fascin-1 transcripts in human carcinoma cell lines demonstrated a decrease in

protrusions and with this, inhibited cell migration (Hashimoto et al., 2007). Furthermore, fascin-1 upregulation in H1299 epithelial-like cells and colonic epithelial cells was found to promote invasiveness (Jawhari et al., 2003; Pan et al., 2017). This invasive property is conferred upon such tumour cells by epithelial-mesenchymal transition (EMT), which is promoted by fascin-1 (Machesky & Li, 2010). As such, fascin-1 is today considered a potential therapeutic target. For example, migrastatin is an inhibitor binding to actin-binding sites on fascin-1 – thereby blocking cell migration and metastasis (Chen et al., 2010).

The abundance of fascin-1 is controlled in two ways: transcriptional regulation and post-transcriptional regulation (Hashimoto et al., 2011). For *FSCN1*, a promoter region is located within the -219/+114 region and contains very transcriptionally active motifs to which transcription factors like NFκB can bind (Hashimoto et al., 2009). A variety of cytokines including IL-6 and TNFα have been observed to promote *FSCN1* expression via the NFκB pathway (Liu et al., 2021; Snyder et al., 2014). Indeed, IL-1β was found to promote *FSCN1* expression in Oral Squamous Cell Carcinoma (OSCC) cells (Lee et al., 2018). However, the effects of IL-1α – which binds to the same receptor – seem to have not yet been explored.

As previously mentioned, there are several consequences as a result of upregulated *FSCN1* expression, including EMT and metastatic colonisation (J. Li et al., 2018; Lin et al., 2019; Zhao et al., 2018). Beyond this, upregulation of *FSCN1* expression can promote cancer stem cell self-renewability (via Notch signalling), proliferation (via YAP/TEAD signalling), and chemoresistance by regulating integrin-mediated survival (Barnawi et al., 2016; Ghebeh et al., 2014; Liang et al., 2016). Given that findings suggest ARDS displays a tumour microenvironment in which metastasis is enhanced and fascin-1 mRNA levels are upregulated in NSCLC tissue, further looking into upregulated *FSCN1* expression due to inflammatory cytokines is warranted (Liang et al., 2016; Pooladanda et al., 2023).

2.4. Interleukin 1 α and Inflammation

Interleukin 1 (IL-1) is a family of cytokines abundantly expressed in many cell types and primarily associated with innate immunity (Dinarello, 2018; Voronov, 2013). As such, there are 11 members in the IL-1 family – these can be further divided into 3 subfamilies based on consensus sequences (Dinarello, 2018). The three subfamilies are IL-1, IL-18, and IL-36 (Dinarello, 2018). The IL-1 subfamily specifically includes IL-1 α and IL-1 β , where IL-1 β has been most studied (Dinarello, 2018). Both IL-1 α and IL-1 β bind the same receptor – IL-1R1 together with the IL-1R3 co-receptor – and induce similar biological functions (Dinarello, 2018; Voronov, 2013). Despite these similarities, IL-1 β is only active when processed and secreted, whereas for IL-1 α both the processed and precursor forms are active (Voronov, 2013). Interestingly, IL-1 α is constitutively present in epithelial cells, while IL-1 β is only expressed during disease (Dinarello, 2018). As such, the IL-1 α precursor can be found on the plasma membrane or more often internally cell-bound (Kurt-Jones et al., 1985; Malik & Kanneganti, 2018).

Upon translation, a 31 kDa pro-IL-1 α with a nuclear localisation signal (NLS) is formed (Wessendorf et al., 1993). Under homeostatic conditions, pro-IL-1 α is therefore shuttled to the nucleus (Malik & Kanneganti, 2018). The exact mechanisms involved in nuclear localisations remain unclear (Malik & Kanneganti, 2018). The pro-form of IL-1 α is converted into its 17 kDa active form by caspase-1, but can also be cleaved by chemise, elastase, or granzyme B (Malik & Kanneganti, 2018). Indeed, during inflammatory cell death due to intracellular pathogens like viruses, inflammasomes can activate caspase-1 for pro-IL-1 α cleavage (Malik & Kanneganti, 2018). During necrosis, active IL-1 α then quickly shuttles to the cytosol (Dinarello, 2018). As such, IL-1 α is released and the lysate, therefore, is highly inflammatory with IL-1 α acting as a DAMP (Dinarello, 2018; Malik & Kanneganti, 2018). On the contrary, during apoptosis, IL-1 α will shuttle to the nucleus and bind chromatin, where IL-1 α will then be cleared via apoptotic bodies in a controlled manner (Dinarello, 2018; Malik & Kanneganti, 2018). In this case, inflammation will not be induced.

In combating infection, the innate immune system manifests as inflammation (Dinarello, 2018). However, when out of control, this can be detrimental to the host (Dinarello, 2018). Indeed, IL-1 has been observed to promote tumour metastasis and mutations leading to IL-1 α dysregulation are frequently associated with cancer (Malik & Kanneganti, 2018; Voronov, 2013; Xu et al., 2010). As such, biologics like MABp1 – an IL-1 α -neutralising monoclonal antibody – are currently in clinical trials and have shown improved survival in NSCLC patients (Hong et al., 2015). Since IL-1 α has not been as extensively studied and there are strong associations between inflammation and progression of cancers like NSCLC, conducting further investigation into IL-1 α and cancer metastasis links is warranted.

2.5. A549 Cells as the Cell Line Model

The A549 cell line is an adenocarcinomic alveolar basal epithelial cell line with morphological and biochemical features of ATII cells (Garcia-de-Alba, 2021; B. T. Smith, 1977). As such, the A549 cell line has been extensively used as an NSCLC model (Garcia-de-Alba, 2021). For example, A549 cells demonstrated EMT following a 21-day exposure to inflammatory cytokines (R. Li et al., 2020). EMT is also a key process conferring invasive properties to tumour cells, as mentioned previously, and is furthermore promoted by fascin-1 (Machesky & Li, 2010). Since *FSCN1* is expressed in A549 cells (nTPM=108.3), the A549 cell line is an adequate model for this investigation (The Human Protein Atlas, n.d.). As such, A549 cells will be used as an NSCLC model to look at the effects of IL-1 α treatment as observed in ARDS on the change in *FSCN1* expression. In the larger context, further investigations into changes in *FSCN1* expression as a result of acute inflammation may provide further insights into the possible promotion of metastasis in NSCLC due to acute inflammation.

2.6. Aim

This study aims to determine the effect of IL-1 α treatment on *FSCN1* expression in the A549 cell line as a model for non-small cell lung cancer (NSCLC).

2.7. Objectives

1. Extraction of adequately pure, concentrated and intact RNA from IL-1 α treated or untreated A549 cells.
2. Production of adequate cDNA by reverse transcription.
3. Design of adequate *FSCN1* primers and determination of an appropriate annealing temperature.
4. Determining the change in *FSCN1* expression upon treatment of A549 cells with IL-1 α .

2.8. Hypothesis

Treatment of A549 adenocarcinomic human epithelial cells with the pro-inflammatory cytokine IL-1 α will upregulate *FSCN1* expression.

3. METHODS

3.1. Cell Culture Treatment

A549 cells (CCL-185) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The A549 cells were cultivated at 37 °C and 5% CO₂ to 80% confluency by in-house technicians in T75 flasks with Dulbecco's Modified Eagle Medium (DMEM), 1% penicillin and streptomycin, 1% glutamine, 10% fetal bovine serum (FBS), and 1% non-essential amino acids (NEAA). Cells were washed in phosphate buffered saline (PBS) and incubated (18 h at 37 °C and 5% CO₂) with DMEM and 0.01% bovine serum albumin (BSA). This was followed by treatment of the cells with either only DMEM (control) or DMEM supplemented with 2.5 ng/ml IL-1 α (200-LA/CF) (Bio-Techne, Minneapolis, MN, USA) and incubation for 48 h at 37 °C and 5% CO₂. PBS pre-washed cells were harvested using the scraping technique, followed by aliquoting, drying, and freezing at -80 °C.

3.2. RNA Extraction and Quality Control

IL-1 α treated and untreated A549 cells (section 3.1) were homogenised and underwent RNA extraction and purification using the RNeasy[®] Mini Kit (74104) (Qiagen, Hilden, Germany) according to manufacturer instructions and the following adaptations. Buffer RLT (350 μ L) was added to each of two control and two IL-1 α treated A549 cell pellets due to a cell count of $<5 \times 10^6$ as determined by in-house technicians. Following the addition of 70% ethanol (350 μ L) to each control and treated sample, A549 cells were homogenised via vigorous pipetting. Control and treated samples were each transferred to an RNeasy spin column and centrifuged for 15 s at 14,000 rpm. A DNase digestion step using diluted DNase I (136 μ g/mL) was carried out using the RNase-Free DNase Set (79254) (Qiagen, Hilden, Germany). Buffer RW1 wash steps were carried out with centrifugations for 15 s at 14,000 rpm. The first buffer RPE wash step was carried out with centrifugation for 15 s at 14,000 rpm. The second buffer RPE wash step was carried out with centrifugation for 2 min. at 14,000 rpm. Subsequent centrifugation was carried out for 1 min. at 14,000 rpm. The same collection tube was reused throughout the buffer RPE washing steps. RNase-free water was added to the RNeasy spin columns and centrifugation was carried out for 1 min. at 14,000 rpm. The elute was passed through the respective RNeasy spin columns a second time with centrifugation for 1 min. at 14,000 rpm to obtain the RNA extracts.

RNA concentration and purity were determined using the NanoDrop[™] Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) by taking 1 μ L from each of the four RNA extract samples. RNA integrity was determined by running the two IL-1 α treated and two untreated RNA extracts on a 1.5% (w/v) agarose and 1x TAE buffer gel with added SafeView[™] nucleic acid dye (5 μ L) (NBS-SV1) (NBS Biologicals, Huntington, UK).

5x blue loading dye (2 μ L) (BIO-37045) (Bioline Ltd., London, UK) was added to each of the four RNA samples. The four RNA samples were then loaded onto the gel (12 μ L each) together with the 1 kb DNA HyperLadder[™] (5 μ L) (BIO-33053) (Bioline Ltd., London, UK). The gel was run at 100 V for 50 min. and for all four RNA samples, the 28S and 18S amplicons were compared via densitometric analysis

using Fiji ImageJ. Densitometric analysis was chosen as a quantitative method to compare amplicon brightness in a more objective manner using statistical analysis.

3.3. Reverse Transcription

One suitable sample in terms of concentration and purity was selected for reverse transcription from both the control and treated RNA extracts. Reverse transcription was carried out using the High-Capacity RNA-to-cDNA™ Kit (4387406) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to manufacturer instructions. The reverse transcription reaction mixture (20 µL) (table 3.1) for the four RNA extract samples was then transferred to a thermal cycler with the following program: 1 h at 37.0 °C, then 5 min. at 95.0 °C, and finally resting at 4.0 °C until further processing.

Table 3.1 – Components of the reverse transcription reaction mixture for the selected control and IL-1a treated samples as per manufacturer instructions. RNA extract volumes were <9 µL at 2 µg RNA given their respective RNA concentrations.

	Positive (RT+) Control 2	Negative (RT-) Control 2	Positive (RT+) IL-1α Treated 2	Negative (RT-) IL-1α Treated 2
2x RT Buffer Mix (µL)	10	10	10	10
20x RT Enzyme Mix (µL)	1	<i>None</i>	1	<i>None</i>
RNA Extract (µL)	3.6	3.6	5.8	5.8
Nuclease-Free Water (µL)	5.4	6.4	3.2	4.2
Total (µL)	20	20	20	20

3.4. Primer Design

Primers (forward and reverse) for *FSCN1* (NM_003088.4) (table 3.2) were designed using NCBI PrimerBLAST and submitted to Eurofins Genomics (Ebersberg, Germany) for synthesis. β-Actin was selected as the housekeeper due to its abundant (nTPM=3784.7) and constitutive expression in A549 cells (The Human Protein Atlas, n.d.). In-house sourced forward and reverse primers for *ACTB* (NM_001101.5) (table 3.2), which encodes β-actin, were used.

Table 3.2 – Forward and reverse primer sequences for *FSCN1* and *ACTB* are shown following PrimerBLAST analysis. Melting temperatures (T_m) and amplicon size is also shown for each primer design.

Primer	Sequence	Melting Temperature (T_m) (°C)	Amplicon Size (bp)
<i>FSCN1</i> Forward	5'-GCGCCTACAACATCAAAGACTC-3'	59.91	220
<i>FSCN1</i> Reverse	5'-TAGTACTCCCAGAGCGAGGC-3'	60.47	
<i>ACTB</i> Forward	5'-GGGAAATCGTGCGTGACATT-3'	57.30	202
<i>ACTB</i> Reverse	5'-CCACAGGACTCCATGCCC-3'	60.50	

3.5. End-Point PCR

The expression of the housekeeper gene for β -actin was used to normalise *FSCN1* expression. For the polymerase chain reaction (PCR), one non-template control (NTC) and four cDNA samples (section 3.3) were prepared. A master mixture was prepared for the five total reactions and one extra for safety (table 3.3). To each of the five reaction tubes, 9 μ L of the master mixture was added.

Table 3.3 – The master mixture was set up for 5 reactions and 1 extra for safety. *ACTB* primers were added to the reaction mixture. No cDNA was added at this stage.

Reagent	Volume per 6 Reactions (μ L)
2x Biomix Reagent	30
Forward Primer (100 pmol/ μ L)	6
Reverse Primer (100 pmol/ μ L)	6
Nuclease-Free Water	12
Total	54

To each of the four PCR reaction tubes, 1 μ L cDNA was added. No cDNA was added to the NTC reaction tube and 1 μ L nuclease-free water was added instead. All five PCR samples were vortexed and placed into a thermal cycler with the following program: 15 s at 94.0 °C, then 15 s at 56.0 °C, and finally 20 s at 72.0 °C (35 cycles). A 1.5% (w/v) agarose and 1x TAE buffer gel with added SafeView™ nucleic acid dye (5 μ L) (NBS-SV1) (NBS Biologicals, Huntington, UK) was used. 5x blue loading dye (BIO-37045) (Bioline Ltd., London, UK) was added to each of the five PCR samples. The five PCR samples were loaded on the 1.5% (w/v) agarose and 1x

TAE buffer gel (12 μ L) together with the 1 kb DNA HyperLadder™ (5 μ L) (BIO-33053) (Bioline Ltd., London, UK). The gel was run at 100 V for 50 min. and the resulting products were compared via densitometric analysis using Fiji ImageJ. Densitometric analysis was chosen as a quantitative method to compare amplicon brightness in a more objective manner using statistical analysis.

To determine the ideal annealing temperature (T_a) for the *FSCN1* primers, a gradient was established on a 1.5% (w/v) agarose and 1x TAE buffer gel over four temperatures close to the reported melting temperature by the manufacturer. The four melting temperatures were: 52.0 °C, 54.0 °C, 56.3 °C, and 58.4 °C. As such, a master-mix for five PCR samples with one added sample for safety was prepared as explained above for β -actin (one NTC and four cDNA samples). Since the IL-1 α treatment group is expected to display increased *FSCN1* expression, only the IL-1 α treated positive reverse-transcribed cDNA was tested at each of the four temperatures. Instead of β -actin forward and reverse primers, *FSCN1* forward and reverse primers were added. The 1.5% (w/v) agarose and 1x TAE buffer gel was prepared, loaded, and run as described above for β -actin. The appropriate melting temperature was selected based on amplicon intensity for the four temperature groups.

To determine the change in *FSCN1* expression, both the negative and positive reverse-transcribed cDNA samples for the control and IL-1 α treated groups were used. The added cDNA used was prepared as described in sections 3.2 and 3.3 (n=1). The cDNA samples underwent PCR and were run on a 1.5% (w/v) agarose and 1x TAE buffer gel as previously described for β -actin. A further four IL-1 α treatment groups with their respective controls were tested using cDNA preparations from colleagues in our laboratory (n=2,3,4,5).

3.7. Statistical Analysis

Normalised densitometric data from the End-Point PCRs (n=1,2,3,4,5) were compared using an unpaired two-tailed t-test since treatment groups are independent and ratio data is collected. Furthermore, the data were tested for normal distribution using the Shapiro-Wilk test – $P>0.05$ was deemed as a normal

distribution. $P < 0.05$ was deemed statistically significant. Analyses were carried out using R (R Core Team, 2021) and RStudio (RStudio Team, 2021). The package 'ggplot2' was used to generate any graphs (Wickham, 2016).

4. RESULTS

The aim of this investigation is to determine the effects of IL-1 α treatment of A549 cells on *FSCN1* expression. To do this, pure and intact extracted RNA needed to be reverse transcribed. Following this, *FSCN1* primer annealing temperatures were optimised. Lastly, *FSCN1* expression was normalised using *ACTB* expression levels in order to determine a change in *FSCN1* expression following IL-1 α treatment.

4.1. Extracted RNA Quality and Purity

The objective in analysing the extracted RNA here is to determine purity, concentration and integrity of the RNA. This is necessary so that the RNA can be successfully reverse-transcribed. For the two A549 untreated control cell pellets – control 1 and control 2 – the 28S:18S ratios were determined to be 1.59:1 and 1.90:1 respectively. For the two A549 IL-1 α cell pellets – treated 1 and treated 2 - the 28S:18S ratios were determined to be 1.63:1 and 1.67:1 respectively. Background corrected mean pixel density of amplicons was used to determine 28S:18S ratios. As shown in figure 4.1, control 1 (C1) especially shows smearing and treated 2 (IL1 α 2) shows a reduced pixel density in the 28S amplicon compared to other products. However, all 28S and 18S products are intact and the RNA can be used for further processing.

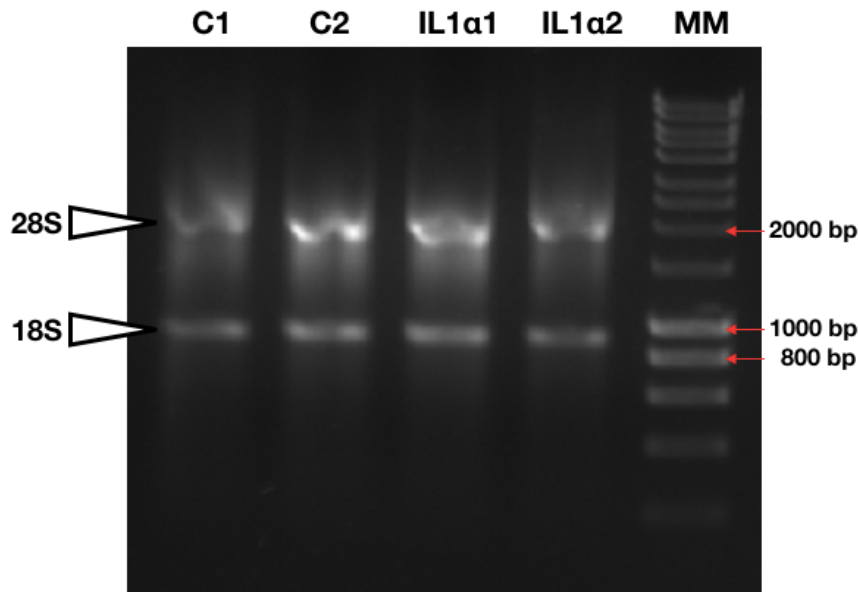


Figure 4.1 – Extracted RNA run on a 1.5% (w/v) agarose and 1x TAE buffer gel showing 28S and 18S products. Two untreated control A549 RNA extracts (C1 and C2) and two IL-1 α treated A549 RNA extracts (IL1 α 1 and IL1 α 2) were tested. A 1 kb DNA HyperLadder™ (MM) was also used. All RNA extract samples show characteristic 28S amplicons at around 2000 bp and 18S amplicons at around 900 bp. Some smearing and differences in amplicon intensity are visible.

As shown in table 4.1, the nucleic acid concentration was high in all four A549 cell pellet extracts. The A260/280 ratios for all four A549 cell pellet extracts also fell close to the ideal 2.0 ratio. All of the A260/230 ratios for the four A549 cell pellet extracts fell short of the ideal 1.8-2.2 ratio. However, in practice, this is to be expected and the four A549 cell pellet extracts can still be used for further processing. The two A549 cell pellet extracts – control 2 and treated 2 – most closely produced the ideal 2:1 (28S:18S) ratio. Furthermore, these A549 cell pellet extracts produced good nucleic acid yields and limited impurities compared to other extracts. As such, control 2 and treated 2 were selected for further processing.

Table 4.1 – RNA concentrations, A260/280 ratio, and A260/230 ratio in each of the untreated control and IL-1 α treated A549 extracts. Control 2 and IL-1 α treatment 1 produced the highest RNA concentrations. However, since IL-1 α treatment 1 also produced a rather low A260/230 ratio, IL-1 α treatment 2 was selected for further processing.

RNA Extract Sample	RNA Concentration (ng/ μ L)	A260/280 Ratio	A260/230 Ratio
Control 1	440.6	2.13	1.26
Control 2	551.9	2.09	1.40
IL-1 α Treatment 1	523.5	2.10	0.96
IL-1 α Treatment 2	345.8	2.12	1.29

4.2. β -Actin Expression in Control and IL-1 α Treated cDNA Samples

The aim of analysing β -actin expression levels as the housekeeper gene using end-point PCR is to ascertain whether the cDNA reverse transcribed from extracted RNA (section 3.3) is suitable for further investigations. Expression levels of β -actin will also be used to normalise *FSCN1* expression. As seen in figure 4.2, β -actin amplicons are clearly visible at approximately the 200 bp mark for both positive control and IL-1 α treated cDNA samples obtained from reverse transcribed RNA (section 3.3). As for densitometric analysis, the mean background corrected pixel density was similar for both positive control and IL-1 α treated cDNA samples at 131.59 and 107.63 respectively. Furthermore, as seen in figure 4.2, no amplicons are visible in the NTC, suggesting no contamination by foreign DNA. Also, no amplicons being visible in both negative control and IL-1 α treated cDNA samples suggests that most residual DNA has been removed upon RNA extraction. As expected, some primer dimerisation would occur and this is visible at the lower base pair region in figure 4.2. As such, the cDNA samples obtained from extracted RNA can be used for further processing and later normalisation of data obtained from the *FSCN1* products.

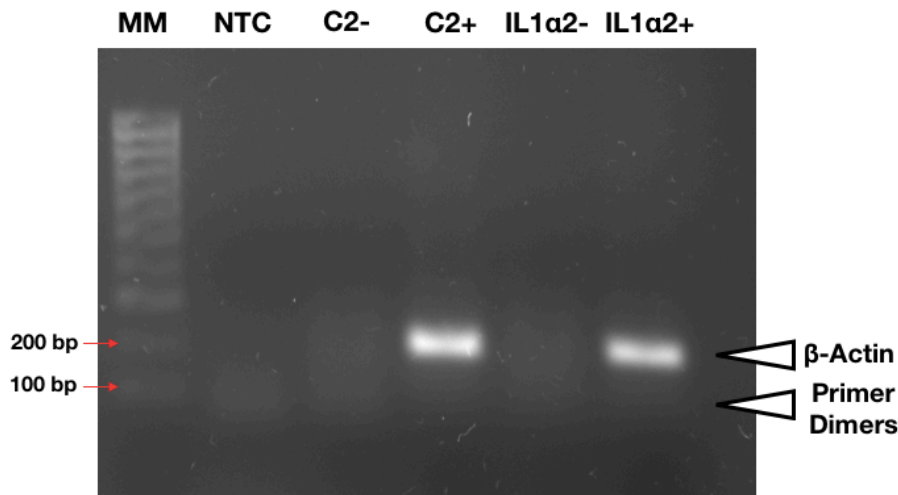


Figure 4.2 – cDNA run on a 1.5% (w/v) agarose and 1x TAE buffer gel showing β -actin amplicons of the selected control and IL-1 α treated A549 extract samples. RNA was reverse transcribed and run on a gel. During the reverse transcription reaction, negative (RT-) control and negative (RT-) IL-1 α treated samples (C2- and IL1 α 2-) did not include reverse transcriptase. A non-template control (NTC) with no added cDNA was also run. A 1 kb DNA HyperLadder™ (MM) was used. As expected, amplicons appeared in C2+ and IL1 α 2+ and not in C2- and IL1 α 2-. No amplicons were visible in NTC as expected. C2+ and IL1 α 2+ β -actin amplicons were visible around 200 bp. Some smearing and β -actin primer dimers were observed.

4.3. *FSCN1* Primers Annealing Temperature Optimisation

Since these *FSCN1* primers have been designed and synthesised for this investigation, an optimal annealing temperature needed to be determined for PCR using *FSCN1* primers. As shown in figure 4.3, amplicons with annealing temperatures set at 52 °C, 54 °C, 56.3 °C, and 58.4 °C were all bright and well-defined. Again, no amplicons in NTC indicate an absence of contamination with foreign DNA. Primer dimers, as expected, were slightly visible around the 100 bp mark. For further processing, 56.3 °C was selected. Upon visual inspection, all amplicons at the tested temperatures indicate that acceptable products have been produced. As such, the choice of 56.3 °C was made for convenience purposes in our laboratory.

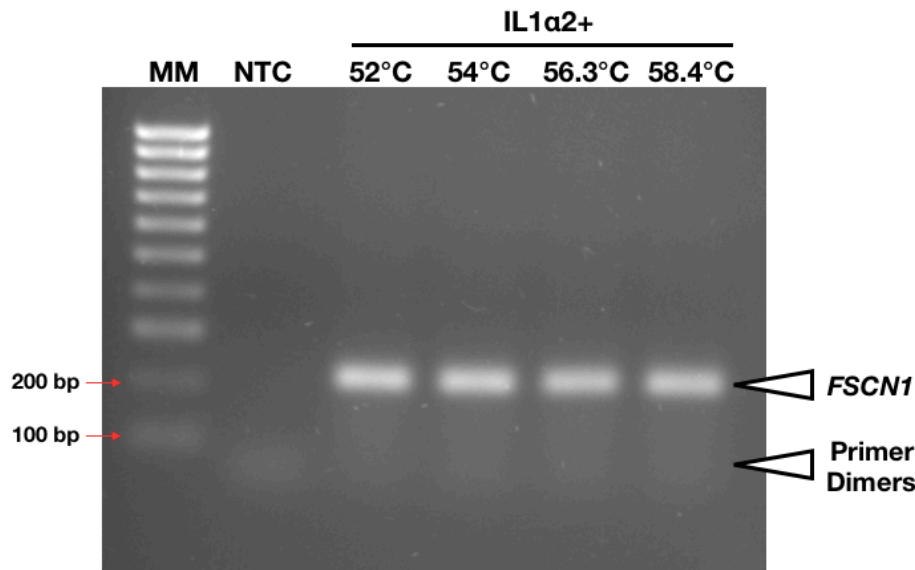


Figure 4.3 – IL-1 α treated cDNA samples put through PCR with *FSCN1* primers at different annealing temperatures. Tested annealing temperatures were chosen based on the manufacturer reports. IL-1 α treated cDNA samples were chosen due to an expected *FSCN1* expression increase. The second positive (RT+) IL-1 α treated cDNA sample (IL1 α 2+) was chosen as cDNA would be present and the RNA quality was most appropriate. The cDNA samples with *FSCN1* primers were run on a 1.5% (w/v) agarose and 1x TAE buffer gel with a 1 kb DNA HyperLadder™ (MM). Almost identical amplicons in terms of brightness can be observed around 200 bp. Smearing is minimal, but primer dimers can be observed.

4.4. Effect of IL-1 α Treatment of A549 Cells on *FSCN1* Expression

The objective here was to determine a change in *FSCN1* expression following IL-1 α treatment and whether such a change is significant. Initial observations of the gel using *FSCN1* primers as shown in figure 4.4 would suggest a slight increase in *FSCN1* expression after IL-1 α treatment. Indeed, densitometric analysis for the gel in figure 4.4 revealed a 0.52-fold increase in normalised and background-corrected mean pixel density.

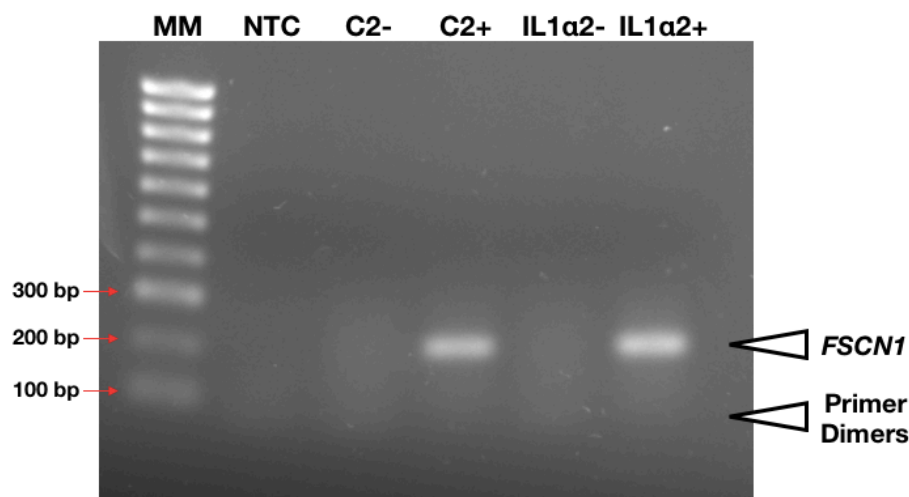


Figure 4.4 – cDNA run on a 1.5% (w/v) agarose and 1x TAE buffer gel showing *FSCN1* amplicons of the selected control and IL-1 α treated A549 extract samples (n=1). Of the two control and IL-1 α treated A549 extracts, the best of each (C2 and IL1 α 2) were selected based on RNA quality. During the reverse transcription reaction, negative (RT-) control and negative (RT-) IL-1 α treated samples (C2- and IL1 α 2-) did not include reverse transcriptase. A non-template control (NTC) with no added cDNA was also run together with a 1 kb DNA HyperLadder™ (MM). As for the positive (RT+) control and positive (RT+) IL-1 α treated samples (C2+ and IL1 α 2+), amplicons can be observed around 200 bp, where the IL1 α 2+ product is slightly brighter than the C2+ product. Smearing is minimal with some primer dimers.

However, later repeats using positive and negative control and IL-1 α treated A549 cDNA from colleagues in our laboratory revealed a mean decrease in normalised and background-corrected mean pixel density from 0.74 to 0.73. As seen in figure 4.5, and indeed many of the other repeat gels using *FSCN1* primers, initial observation suggests brighter amplicons and consequent increased *FSCN1* expression. However, following background correction and normalisation, a decrease in mean pixel density was observed. In figure 4.5, this is presented as a 0.17-fold decrease in normalised and background-corrected mean pixel density.

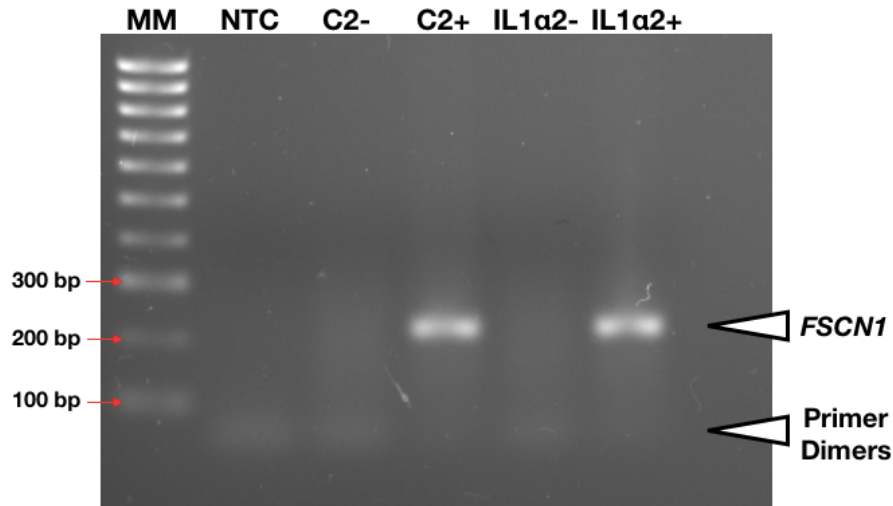


Figure 4.5 - cDNA run on a 1.5% (w/v) agarose and 1x TAE buffer gel showing *FSCN1* amplicons of the selected control and IL-1 α treated A549 extract samples (n=2). Of the two control and IL-1 α treated A549 extracts, the best of each (C2 and IL1 α 2) were selected based on RNA quality. During the reverse transcription reaction, negative (RT-) control and negative (RT-) IL-1 α treated samples (C2- and IL1 α 2-) did not include reverse transcriptase. A non-template control (NTC) with no added cDNA was also run together with a 1 kb DNA HyperLadder™ (MM). As for the positive (RT+) control and positive (RT+) IL-1 α treated samples (C2+ and IL1 α 2+), amplicons can be observed around 200 bp, and the C2+ and IL1 α 2+ amplicons cannot be discerned in terms of brightness. Smearing is minimal with some primer dimers.

Overall, the mean decrease in normalised and background-corrected mean pixel density was minute as seen in figure 4.6. Despite this decrease, many of the repeat groups produced a slight increase as shown in table 4.2. There was no significant difference between the two mean values (unpaired two-tailed t-test, $t = 0.12$, $df = 6.95$, $p = 0.907$), with the control group producing the higher mean normalised and background corrected mean pixel density.

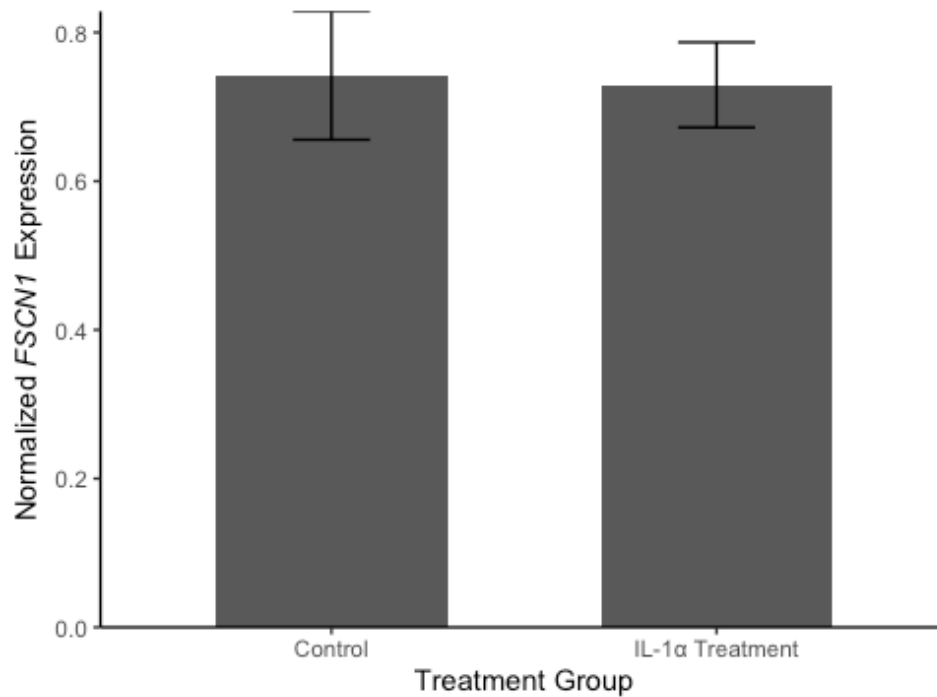


Figure 4.6 – Difference in background corrected and normalised mean pixel density of *FSCN1* products in untreated control and IL-1 α treated cDNA samples on 1.5% (w/v) agarose and 1x TAE buffer gels. A total of five (n=5) agarose gels were included, where four of the cDNA samples were produced by colleagues in our laboratory. Standard deviation error bars were applied. Contrary to expectation, there was a slight decrease in *FSCN1* expression across the cDNA samples. As also seen by the lacking overlap in error bars, the difference was not significant.

Table 4.2 – The β -actin-normalised *FSCN1* expression and fold difference of the selected samples in each repeat group. For n=3, a colleague in our laboratory from whom the samples were obtained determined RNA quality of their control 1 to be higher than control 2. As such, control 1 was used to produce cDNA. To normalise *FSCN1* expression, *ACTB* amplicon mean pixel density was divided by *FSCN1* mean pixel density. The difference was determined in terms of the control.

Repeat (n)	Sample	Normalised <i>FSCN1</i> Expression	Normalised <i>FSCN1</i> Expression Fold Difference
1	Control 2	0.48	0.52-Fold Increase
	IL-1 α Treatment 2	0.73	
2	Control 2	1.00	0.17-Fold Decrease
	IL-1 α Treatment 2	0.83	
3	Control 1	0.82	0.32-Fold Decrease
	IL-1 α Treatment 2	0.56	
4	Control 2	0.65	0.01-Fold Increase
	IL-1 α Treatment 2	0.66	
5	Control 2	0.76	0.15-Fold Increase
	IL-1 α Treatment 2	0.87	

5. DISCUSSION

The purpose of this study was to determine the effects of IL-1 α treatment on *FSCN1* expression in A549 cells as a model for non-small cell lung cancer. RNA was extracted from A549 cells that were either treated with IL-1 α or left untreated. For further processing, extracted RNA was analysed for purity, integrity and concentration using 28S:18S ratio comparison, and also A260/280 and A260/230 ratios. For use in PCR and gel electrophoresis, extracted RNA was reverse transcribed to cDNA. Integrity of the cDNA for further processing was determined by analysing expression levels of the constitutively expressed housekeeper *ACTB* (β -actin) via end-point PCR and gel electrophoresis. End-point PCR and gel electrophoresis were used to identify changes in *FSCN1* expression levels. Analysis of amplicons on the electrophoresed agarose gel products was then carried out by densitometric analysis to obtain quantitative data for statistical analysis. Normalisation of *FSCN1* amplicon densitometric data using *ACTB* amplicon

densitometric data produced results showing a small decrease in *FSCN1* expression in IL-1 α treated compared to untreated A549 cells.

When run on an agarose gel, the extracted RNA samples (section 3.2) produced characteristic 28S and 18S amplicons as shown in figure 4.1. To serve as a template for reverse transcription, the RNA needs to be intact. A 28S:18S ratio of 2:1 is generally considered ideal when determining the level of RNA degradation (Fleige & Pfaffl, 2006). The 28S:18S ratios of control 2 (C2) and treated 2 (IL1 α 2) were 1.90:1 and 1:67:1 respectively. These ratio values were closer to the ideal 2:1 than those of control 1 (C1) and treated 1 (IL1 α 1). As such, control 2 (C2) and treated 2 (IL1 α 2) were selected for further processing in section 3.3. Despite this, especially the 1:67:1 ratio is still far from the ideal 2:1. In some cases, a ratio lower than 1.8:1 may even be regarded as too degraded (Fleige & Pfaffl, 2006). Inspection of figure 4.1 also shows visible smearing and some degradation in 28S amplicons. This smearing could be attributed to improper pouring of the agarose gel. Generally, moderate RNA degradation will not have adverse effects on the reverse transcription output (Fleige & Pfaffl, 2006). However, it cannot be ruled out that moderate degradation may affect further processing. As RNA is degraded, *FSCN1* expression levels later determined via end-point PCR may diminish (Gingrich et al., 2006). This may lead to erroneous conclusions when comparing control 2 (C2) and treated 2 (IL1 α 2) – which differ in RNA integrity (Gingrich et al., 2006). As for the A260/280 ratios, a ratio of 2.0 for RNA is considered ideal according to NanoDropTM Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) user instructions. As shown in table 4.1, all control and IL-1 α treatment samples fell close to the ideal 2.0 ratio. A reason for such a deviation could be protein contamination from the RNA extraction step (section 3.2) (Fleige & Pfaffl, 2006). The A260/230 ratios should ideally fall within the 1.8-2.2 range according to NanoDropTM Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) user instructions. Table 4.1 shows all control and IL-1 α treatment samples fell short of this ratio range, which suggests contaminants such as buffers used for RNA extraction (section 3.2). These contaminants may interfere with reaction co-factors such as Mg²⁺ or Mn²⁺ needed by the reverse transcriptase, or later interactions between cDNA and the gel (Fleige & Pfaffl, 2006).

The *ACTB* (β -actin) amplicons appeared, as expected, in the positive (RT+) control and positive (RT+) IL-1 α treated samples in figure 4.2. Amplicons are clearly visible, indicating that reverse transcription of RNA was successful and intact cDNA was produced. Furthermore, stable expression of *ACTB* as a housekeeper gene indicates normal functioning of gene expression (Eisenberg & Levanon, 2013). Visibility of positive (RT+) control and positive (RT+) IL-1 α treated sample *ACTB* amplicons was also consistently clear across the four remaining repeat groups. As such, the cDNA obtained from four colleagues in our laboratory using the same methodology was intact. However, some of these cDNA samples were low in quality due to contaminations made visible on the agarose gel run with *ACTB* primers. These contaminants appeared in negative (RT-) control and negative (RT-) IL-1 α treated samples at approximately 200 bp. Since *ACTB* amplicons also appear at approximately 200 bp, it is not clear whether positive (RT+) control and positive (RT+) IL-1 α treated samples were also affected by contamination. For these positive samples, the bright *ACTB* amplicons made any potential contaminations difficult to discern. The 200 bp area of negative (RT-) control and negative (RT-) IL-1 α treated samples were used in the background correction of the positive (RT+) samples. Therefore, such contamination could have an impact on the validity of the densitometric analysis. Data on *ACTB* expression for all four remaining repeat groups was available; however, data on quality of the original RNA extracted was not available. Since *ACTB* amplicons are indicative of intact cDNA, data on quality of RNA used to synthesise the cDNA may not be needed. As mentioned previously, however, RNA degradation may affect cDNA synthesis and therefore erroneously decrease amplicon brightness on the agarose gel.

The annealing temperature (T_a) of the *FSCN1* primers was optimised to avoid inefficient binding (T_a too high) or non-specific binding (T_a too low) (Borek, n.d.). As shown in figure 4.3, the *FSCN1* primers produced clear amplicons at all tested annealing temperatures. Given that all amplicons line up clearly at approximately 200 bp, the primers were determined to specifically amplify *FSCN1*. For convenience purposes in our laboratory, 56.3 °C was then selected as the annealing temperature. However, for a more precise determination of a suitable annealing temperature, the amplicons should be compared using densitometric analysis. Looking at several of the agarose gels using either *FSCN1* or *ACTB* (figures 4.2-4.5), primer dimers are

always visible in the higher base pair regions. These primers may be hairpins, self-dimers or cross-dimers (Yang et al., 2020). Primer dimers can lead to erroneous results, where the target gene may not be sufficiently amplified (Yang et al., 2020). As such, the target gene may falsely appear to be less expressed. Primer dimers may be reduced by lowering the concentration of primers when adding these to the reaction mixture.

Fascin-1, encoded by *FSCN1*, is an actin-binding protein and promotes tumour cell migration and metastasis by supporting cellular structures like filopodia (Darnel et al., 2009; Hashimoto et al., 2005, 2011). Multiple studies have observed increases in *FSCN1* expression in epithelial cell lines following IL-1 β treatment (Lee et al., 2018; Zhang et al., 2018). Despite structural differences, IL-1 β and IL-1 α bind and activate the same receptor with very similar cellular responses (Eislmayr et al., 2022). The pro-inflammatory IL-1 α is a cytosol localised cytokine during necrosis and is therefore released during epithelial cell injury as observed in ARDS (Dinarello, 2018; Matthay et al., 2019). As initially hypothesised, *FSCN1* would be upregulated by IL-1 α treatment in A549 cells as a model for non-small cell lung cancer. Surprisingly, as shown in figure 4.6, a mean decrease in *FSCN1* expression was observed. However, this difference was found not to be significant (unpaired two-tailed t-test, $t = 0.12$, $df = 6.95$, $p = 0.907$). Additionally, despite an overall decrease, out of the five repeat groups most produced a small increase in *FSCN1* expression as shown in table 4.2. At this point, it should be mentioned that five repeat groups ($n=5$) are very low, providing only weak statistical power. For future work, the number of repeats should be significantly increased in order to correctly accept or reject the null hypothesis. It is worth noting that transient overexposure to IL-1 α has been observed – albeit in fibrosarcoma models - to have anti-tumourigenic effects (Douvdevani et al., 1992). IL-1 α can indeed have both tumour-promoting and suppressing effects, and this depends on the type of cancer (Baker et al., 2019). A prior investigation looked at *FSCN1* expression changes in oral squamous cell carcinoma cells following treatment for 8 h with 10 ng/mL IL-1 β (Lee et al., 2018). Therefore, future work may look at the effects of IL-1 α treatment at high concentrations on *FSCN1* expression in A549 cells over a shorter period. Furthermore, to avoid ambiguities associated with end-point PCR, quantitative PCR may be a better option for any future work. The advantage of quantitative PCR is its greater detection sensitivity

and ability to track the reaction process in real-time (C. J. Smith & Osborn, 2009). In this investigation, densitometric analysis cannot directly measure *FSCN1* amplification and will therefore be ambiguous.

Going forward, it would also be worth looking at intracellular IL-1 α and its effect on *FSCN1* expression. IL-1 α has dual function in that it can serve as a transcription factor (Dinarello, 2018). Werman et al. (2004) have previously found that induced overexpression of intracellular IL-1 α plays a role in the genesis of inflammation by augmenting proinflammatory genes. Therefore, *FSCN1* may be a target of intracellular IL-1 α as a transcription factor. Other cell lines as models for different types of cancer, such as oral squamous cell carcinoma cells (head and neck cancer) and SW1417 (colorectal cancer) should also be investigated in terms of IL-1 α effects on *FSCN1* expression (Bugshan & Farooq, 2020; Ronen et al., 2019). Should future investigations conclude that extracellular or intracellular IL-1 α does have an effect on *FSCN1* expression, looking at the effects on cell migration would then be the next step. Cell wound closure or scratch assays could be employed to investigate whether the extent of change in *FSCN1* expression due to IL-1 α would actually affect cell migration (Justus et al., 2014). Transgenic animal models may then be employed to look at IL-1 α effects on *FSCN1* expression in congruency with the effects of a change in *FSCN1* expression on cell migration and metastasis in lung cancer.

Necrotic cell death of ATI/II epithelial cells characteristic of ARDS releases DAMPs, where the pro-inflammatory IL-1 α can function as a DAMP (Dinarello, 2018; Matthay et al., 2019). Changes in *FSCN1* expression induced by various cytokines associated with inflammation, such as IL-1 β , IL-6 and TNF α (Lee et al., 2018; Snyder et al., 2014). Increased expression of *FSCN1* is also associated with metastatic colonisation, cell migration and invasion, cell proliferation and epithelial-mesenchymal transition (J. Li et al., 2018; Liang et al., 2016; Lin et al., 2019; Zhao et al., 2018). In conclusion, a minor decrease in *FSCN1* expression was observed following treatment of A549 cells as a model for non-small cell lung cancer with IL-1 α . This decrease was not significant (unpaired two-tailed t-test, $t = 0.12$, $df = 6.95$, $p = 0.907$) in this investigation. However, future studies should investigate shorter bursts of IL-1 α exposure (10 ng/mL for 8 h) to mimic the necrotic environment

in ARDS. As a current potential therapeutic target for various cancer types, further exploration of how *FSCN1* expression is affected is warranted.

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